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A new family of outwardly rectifying potassium channel proteins with two pore domains in tandem

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POTASSIUM channels catalyse the permeation of K^+ ions across cellular membranes and are identified by a common structural motif, a highly conserved signature sequence of eight amino acids in the *P* domain of each channel's pore-forming α-subunit[1,2]. Here we describe a novel K^+ channel (TOKl) from *Saccharomyces cerevisiae* that contains two *P* domains within one continuous polypeptide. *Xenopus laevis* oocytes expressing the channel exhibit a unique, outwardly rectifying, K^+ -selective current. The channel is permeable to outward flow of ions at membrane potentials above the K^+ equilibrium potential; its conduction-voltage relationship is thus sensitive to extracellular K^+ ion concentration. In excised membrane patches, external divalent cations block the channel in a voltage-dependent manner, and their removal in this configuration allows inward channel current. These attributes are similar to those described for inwardly rectifying K^+ channels[3,4] , but in the opposite direction, a previously unrecognized channel behaviour. Our results identify a new class of K^+ channel which is distinctive in both its primary structure and functional properties. Structural homologues of the channel are present in the genome of *Caenorhabditis elegans*.

The peptide sequence of the *P* domain from several K^+ channel proteins was used to search gene databases stored at the National Center for Biotechnology Information (NCBI) using the BLAST sequence alignment program[5]. Along with many known K^+ channel genes, one cosmid from the *S. cerevisiae* genome sequencing project (chromosome X) was identified[6]. Translation of one extended open reading frame (ORF J0911) reveals a hypothetical protein with two *P*-like domains, each containing an octapeptide similar to the K^+ channel signature sequence (Fig. 1*a*). Hydropathy analysis shows that both *P*-like domains are bounded by hydrophobic segments and distinguishes eight potential transmembrane helices (Fig. 1*b*). Marked sequence homology is apparent between the hydrophobic segments following each *P*-like domain and the S6 domains of known K^+ channels (Fig. l*c*). The gene was isolated from yeast genomic DNA by polymerase chain reaction (PCR). Northern blot analysis confirmed that a transcript of 2.2 kilo bases (kb), the expected size for ORF J0911, is present in total yeast RNA and reverse transcription (RT)-PCR was used to delineate the 3' end of the transcript (Fig. 1, methods). Complementary RNA was transcribed from this putative yeast K^+ channel gene (*TOK1*) and injected into *Xenopus laevis* oocytes.

One day after cRNA injection, an outwardly rectifying and non-inactivating current, not present in uninjected cells, was measured by two-electrode voltage clamp (Fig. 2*a*). Unlike voltage-gated K^+ channels, which are activated by membrane depolarization over a fixed voltage range to pass outward current, the voltage at which TOK1-induced currents are first observed changes in response to external K^+ ion concentration (Fig. 2h). The activation potentials of TOK1 currents, defined as the voltage where the slope conductance increases noticeab1l, are –94 ± 4 mV in 2 mM KCl, –46 ± 6 mV in 20 mM KCl, and –2 ± 4 mV in 100 mM KCl (n = 10 oocytes; Fig. 2), close to the K + equilibrium potential (EK) predicted for each solution by the Nernst equation. These changes in activation potential are also apparent in normalized conductance-voltage plots (Fig. 2*c*), indicating that shifts cannot be attributed to driving force induced changes in TOK1 current magnitude. That only minimal inward currents are observed with 20 or 100 mM external KCl at negative voltages (even though there is outward current in 2 mM KCl) supports the premise that the effect of voltage is not primarily intrinsic to TOK 1 (Fig. 2*b*).

Whole-cell TOK1 currents are selective for K^+ over both Na^+ and Cl^-. The magnitude of TOKI currents and the voltage at which they develop are insensitive to substitution of chloride by aspartate (Fig. 2*d*, n = 6) and to substitution of sodium by *N*-methylglucamine (n = 5; Fig. 2, methods). Reversal potentials under pseudo-bi-ionic conditions[8] indicate a permeability ratio (P_K/ P_{Na}) of at least 20 (Fig. 2*e*). TOKI currents are inhibited by

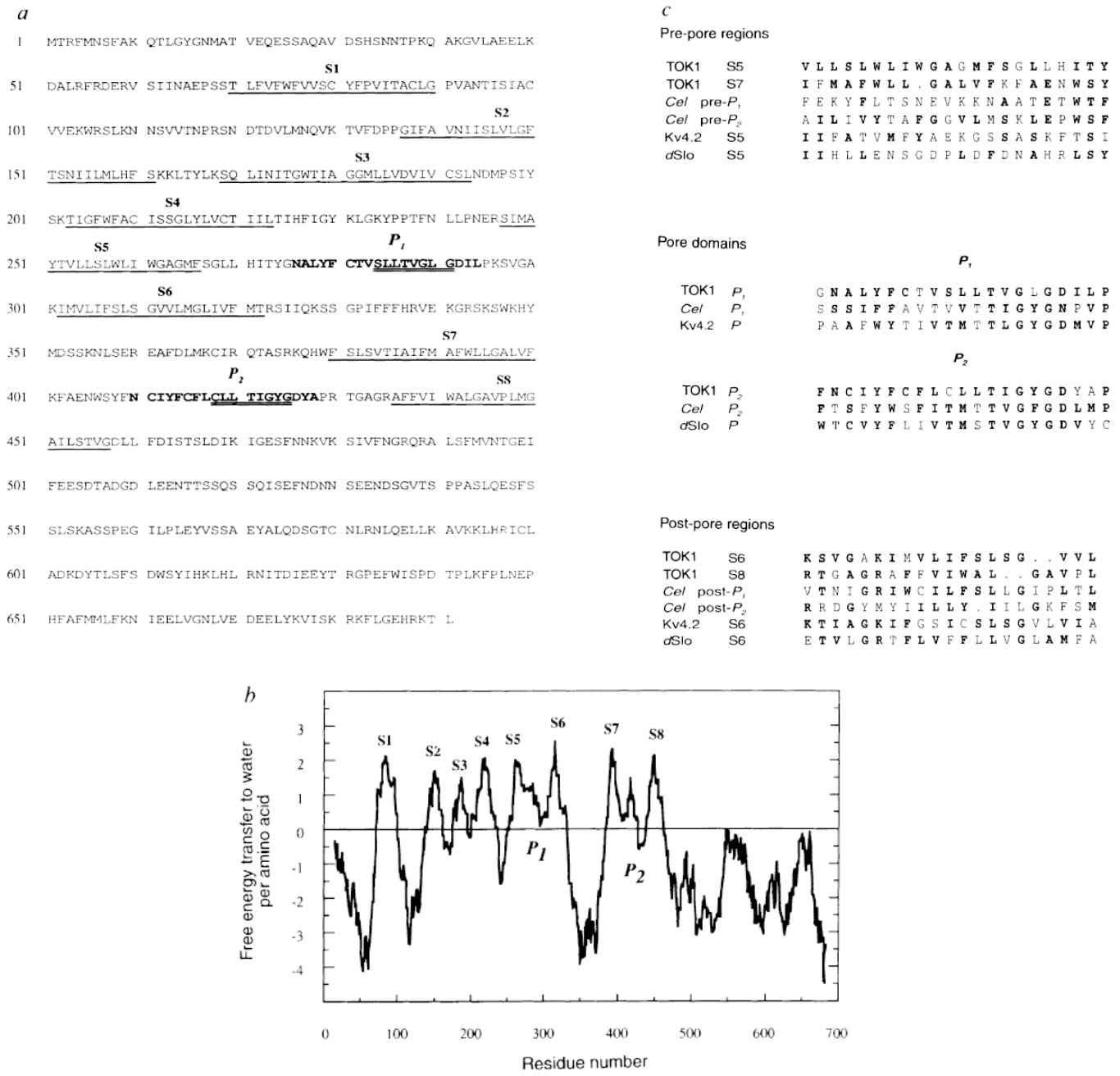


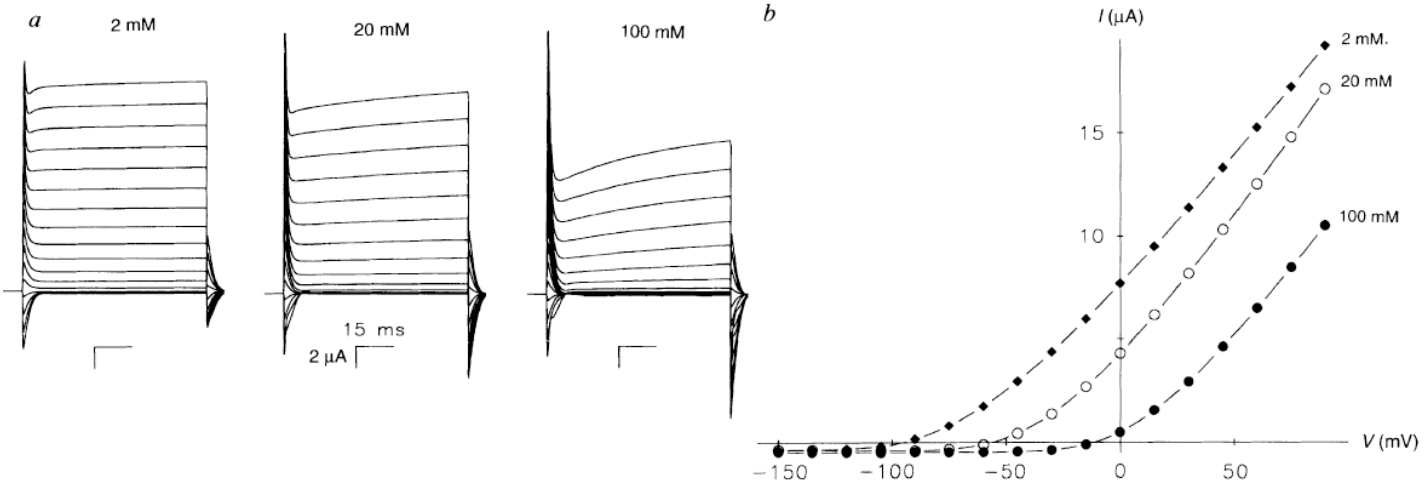
FIG. 1 Primary structure of the TOK1 protein, its hydropathy profile and alignment with related sequences. *a,* The predicted protein sequence of yeast ORF J0911 (accession #X77087). Segments homologous to the *P* domains of known K^+ channels are bold, and residues corresponding to the K^+ channel signature sequence (TXXTXGYG)[2] are double underlined. Hydrophobic segments are labelled S1-S8. *b,* Hydropathy plot of the predicted protein sequence. Average free energy of transfer to water was calculated per amino acid with a window of 20 amino acids[21]. Eight potential transmembrane helices and the two *P* domains are designated. *c,* Alignment of each TOK1 *P* domain and its flanking residues with known K^+ channels and a predicted ORF from *C. elegans* (Cel). Residues identical or similar to TOK1 are bold. BLAST analysis shows the P_1-S6 regions of TOK1 to be 40% identical and 70% similar to the *P*-S6 regions of the mammalian voltage-gated K^+ channel Kv4.2 (# M59980), and the S7-P_2-S8 regions of TOK1 to be 32% identical and 50% similar to the S5-P-S6 regions of the *Drosophila* Ca^{2+}-activated K^+ channel dSlo (# M96840). Hypothetical ORFs from *C. elegans* which predict proteins containing two *P* domains are F22B7.7 (# L12018) and F17C8.5 (# Z35719). The predicted sequence of F22B7.7 (*Cel*) has a hydropathy profile similar to TOK1 from S4 to S8 and exhibits 20% identity and 52% sequence similarity over this region. The probabilities that six random sequences would have the amino acid identities shown here are 1×10^{17} for the *P* domains, and 2×10^{5} for the post-*P* domain regions by the method ofJan and Jan[22]; homology is not significant in the pre-*P* domain regions although marked homology is evident between TOK1 S5 and S7. The TOK1 encoding portion of ORF J0911 has been redesignated by the accession number U28005. METHODS. Default parameters were used in the BLAST search which identified ORF J0911. Hydropathy analysis and sequence alignments were performed using GCG software (Genetics Computer Group, Inc.). ORF J0911 was amplified by PCR

from yeast genomic DNA using primers corresponding to nucleotides 3027-3055 and 5101-5121 of cosmid clone 233 (numerical designations correspond to the published sequence and refer to the complementary DNA strand)[6]. Additional nucleotides were included at the 5' end of each primer to construct the restriction sites *Sal*1, BamHI, and Bg*l*II, HindIII on the 5' and 3' ends of the gene, respectively (5'-GCCACCGAAGCTTAGATCTGCTTCGGGTGTTTGTATATCAAAGTGTC-3' and 5' -GCCACCGGTCGACGGATCCATGACAAGGTTCATGAACAGC-3'). The amplified fragment was digested with *Sal* I and Hind III and cloned into pBluescript (Strategene). This construct was sequenced in its entirety and shown to be identical to the published nucleotide sequence of ORF J0911 (ref. 6). Northern blots of total yeast RNA were probed with ^{32}P-labelled cRNA prepared to the complementary DNA strand. 3' RACE (rapid amplification of eDNA ends; GIBCO BRL) from total yeast RNA located the poly(A) tail at nucleotide 2917, 3' to the putative transcription termination/polyadenylation signal previously identified at position 2981 (ref. 6). Whether there are multiple initiation sites for this gene is not yet known. For production of cRNA the gene was transferred to pGEM-A[23]; this construct is identified as pTOK1.

barium and tetraethylammonium with inhibition constants at 0 mV of 0.06 ± 0.01 mM and 5.7 ± 0.7 mM, respectively (n = 5, 2 mM KCI solution at 10 ms) but are insensitive to 200 nM charybdotoxin, 150 nM iberiotoxin and 200 nM dendrotoxin (n = 2, 2 mM KCI solution). These results suggest that cRNA injection leads to expression of a K^+-selective channel that passes outward current at membrane potentials more positive than E_K.

On-cell patch recording confirms that TOK I currents are mediated by ion channels (Fig. 3). The channels are not seen in patches from uninjected oocytes (n = 8 cells, 14 patches) or oocytes injected with three other K^+ channel cRNAs (minK, n = 4 cells, 16 patches; *d*SLO, n = 10 cells, 30 patches; mSLO, n = 55 cells, > 150 patches)[9 11] but are observed in all oocytes injected with 1-5 ng *TOK1* cRNA (n = 140 cells, >350 patches). TOKI channels in on-cell patches pass outward current in response to depolarization (Fig. 3*a*). Channel behaviour appears largely unaltered by patch excision (Fig. 3*b*); channels do not 'run down' and display no apparent requirement for intracellular constituents. Like whole-cell currents, single channel activity is seen to shift with changes in E_K (Fig. 3*c*). That TOK 1 channels are selective for K^+ over Na^+ is confirmed by elimination of channel activity when NaCI is isotonically substituted for internal KCl in the solution bathing inside-out patches (Fig. 3*d*, n = 4). Single TOKI channels have an apparent conductance of roughly 35 pS in symmetrical K^+, and a 'flickery' appearance under these recording conditions (Fig. 3*e*).

The current-voltage (*I-V*) relationship of inward rectifier K^+ channels results from the blocking of outward current by internal Mg^{2+} and polyamines at voltages above E_K (refs 3, 4, 12-15). Removing divalent cations from the external channel face allows inward flux of K^+ ions through TOK1 channels in off-cell patches at hyperpolarized potentials that were previously electrically quiet (Fig. 4*a*, n = 3 cells, 5 patches). Removing



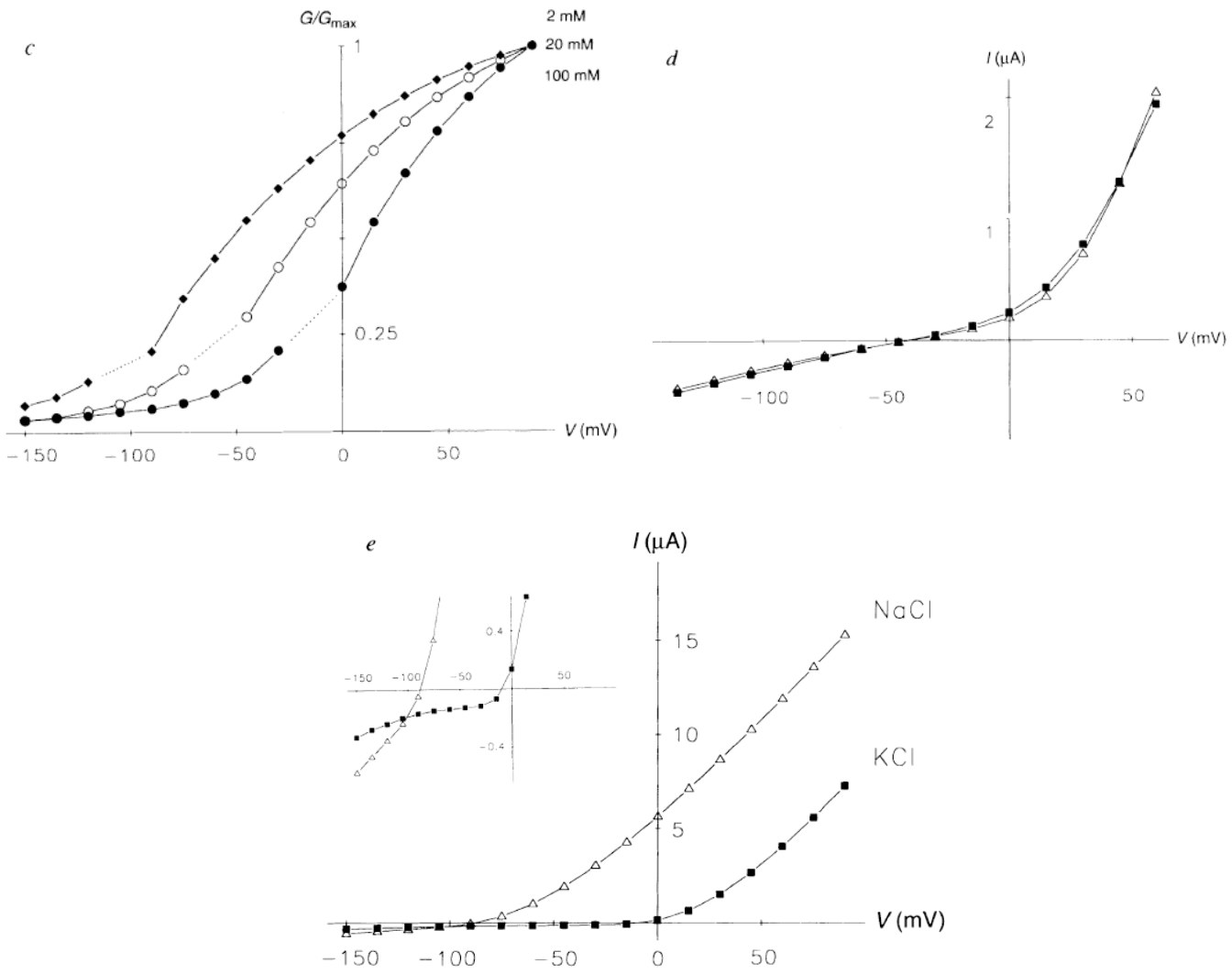


FIG. 2 TOK1-induced currents in *X. laevis* oocytes assessed by two-electrode voltage clamp show outward rectification and activation potentials that shift with external KCl concentration. *a,* Currents elicited by 75 ms pulses from –150 to +90 mV in 15 mV steps from a holding potential of –80 mV with a 1-s interpulse interval and displayed without leak subtraction; bath solution was 2 mM KCl solution, then 20 mM KCl solution, and then 100 mM KCl solution. Scale bars, 2 μA and 15 ms. *b,* Current-voltage relationship at 10 ms into the test pulses shown in *a*; 2 mM KCl solution (filled diamonds), 20 mM KCl solution (open circles) and 100 mM KCl solution (filled circles). *c,* Normalized conductance-voltage relationship at 10 ms into the test pulses shown in a; 2 mM KCI solution (filled diamonds), 20 mM KCI solution (open circles) and 100 mM KCl solution (filled circles); E_K was taken as the reversal potential *a. d,* Currents at 250 ms otherwise as in *a* in 98 mM KCl solution (open triangles) and then 98 mM KAspartate solution (filled squares). *e,* Currents at 10 ms elicited as in a in 100 mM KCI solution (filled squares) and 100 mM NaCl solution (open triangles); the inset shows data points near the reversal potential on an expanded scale. METHODS. cRNA was prepared after linearization of pTOK1 by Notl and transcription with T7 polymerase as described[24]. Transcript concentration was estimated spectrophotometrically and portions stored at -80 oc. *X. laevis* oocytes were isolated and injected with 46 nl cRNA solution containing 1-5 ng transcript as described previously[24]. Whole oocyte currents were recorded 1-4 days after cRNA injection using a two-electrode voltage clamp (Oocyte Clamp, Warner Instruments). Electrodes contained 3M KCI and had resistances of 0.3-1.0 MQ. Recordings were performed under constant perfusion at room temperature. Bath solutions were: 2 mM KCl solution (containing in mM: 98 NaCl, 2 KCl, 1 $MgCl_2$, 0.3 $CaCl_2$, 5 HEPES, pH 7.6); 20 mM KCl solution (in mM: 80NaCl, 20KCl, 1 $MgCl_2$, 0.3$CaCl_2$, 5 HEPES, pH 7.6); 100 mM KCl solution (in mM: 100 KCl, 1 $MgCl_2$, 0.3 $CaCl_2$, 5 HEPES, pH 7.6); 98 mM KCl solution (in mM: 98 KCl, 0.3 $CaCl_2$, 5 HEPES, pH 7 .6); 98 mM KAspartate solution (in mM: 98 KAspartate, 0.3 $CaOH_2$,5 HEPES, pH 7.6); 100 mM NaCl solution (in mM: 100 NaCl, 1 $MgCl_2$,0.3 $CaCl_2$, 5 HEPES, pH 7.6); and 2 mM KCI/98 mM N-methylglucamine (NMG) solution (in mM: 98 NMG, 2 KCl, 1 $MgCl_2$, 0.3 $CaCl_2$, 5 HE PES, pH 7.6).

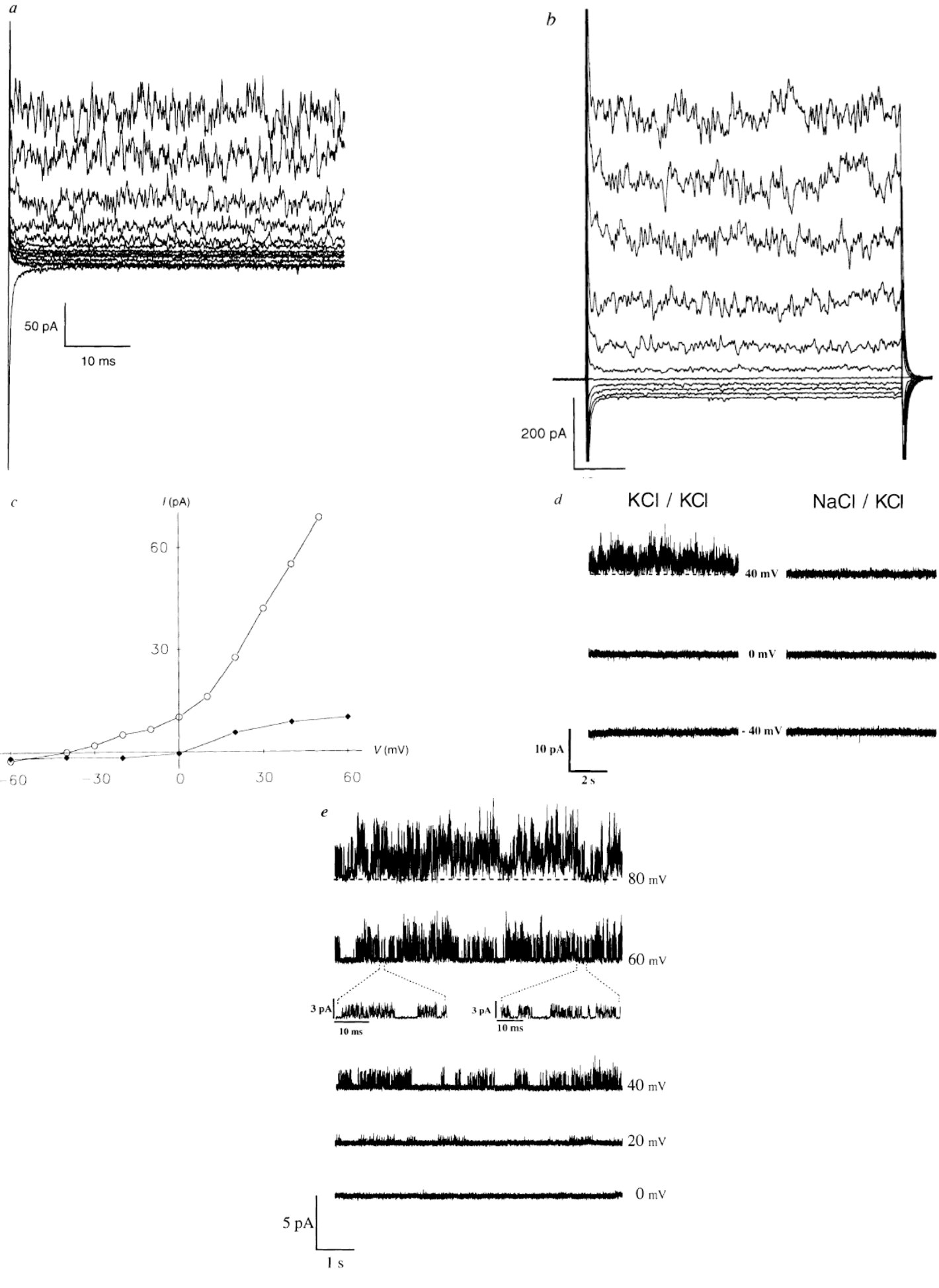

a
50 pA
10 ms
b
200 pA
c
I (pA)
60
30
V (mV)
−60
−30
0
30
60
d
KCl / KCl
NaCl / KCl
40 mV
0 mV
- 40 mV
10 pA
2 s
e
80 mV
60 mV
3 pA
10 ms
3 pA
10 ms
40 mV
20 mV
0 mV
5 pA
1 s

FIG. 3 TOK1 channels in on-cell and excised patches show outward rectification, activation potentials which shift with E_K and K^+ selectivity. *a,* Multiple TOK1 channels recorded in cell-attached mode with 2 mM KCl solution in the bath and pipette (in mM: 2 KCl, 138 NaCl, 1 $MgCl_2$, 5 K EGTA, 10 HE PES, pH 7 .1). Currents were elicited by voltage steps of 10 mV from –60 to +60 mV from a holding voltage of –80 mV. *b,* Multiple TOK1 channels observed in excised inside-out patches in symmetrical 140 mM KCI solution (in mM: 140 KCl, 1 $MgCl_2$, 5 K EGTA, 10 HEPES, pH 7.1). Holding potential was 0 mV and test pulses from –80 to +120 mV in 20 mV increments. *c,* TOK1 channel activity shifts with E_K. The mean current recorded in an excised inside-out patch at command potentials between –60 and +60 mV for 10 s with 14 mM KCl solution in the pipette (in mM: 14 KCl, 126 NaCl, 1 $MgCL_2$, 2 K EGTA, 10 HEPES, pH 7.1) and 14 mM KCl solution (filled diamonds, $E_K \sim 0$ mV) or 140 mM KCl solution (open circles, $E_K \sim -60$ mV) in the bath; the seal resistance was >12 GΩ and currents were not leak subtracted. *d,* Isotonic substitution of internal KCI by NaCl eliminates TOK1 channel activity in inside-out patches: KCl/KCl, symmetrical 140 rnM KCl solution; NaCl/KCl, the bath solution was exchanged for 140 mM NaCl solution (in mM: 140 NaCl, 1 $MgCl_2$, 5 K EGTA, 10 HEPES, pH 7.1). *e,* TOK1 single channel events recorded in an inside-out excised patch with symmetrical 140 mM KCl solution from 0 to 80 mV in 20 mV increments.
METHODS. Patches were made using firepolished pipettes with resistances of 1.5-2.5 MΩ (Corning 7052, Garner Glass, CA). Experiments were performed using an Axopatch 200A amplifier (Axon Instruments) at room temperature. Data were sampled at 20kHz, filtered at 2 kHz through an ITC-16 A/D converter (lnstrutech Corp, NY) and stored in a Mac Quadra 800 computer or to tape for subsequent analysis.

external divalent cations also results in more outward TOK1 current (Fig. 4*a*). This leads to a roughly linear *I-V* relationship from –90 to +90 m V, even in the presence of Mg^{2+} at the intracellular patch surface (Fig. 4*b*). Returning divalent cations to the external bath solution eliminates inward currents at hyperpolarized potentials, thus restoring outward rectification (Fig. 4). These results support the notion that voltage-dependent blocking events contribute to outward rectification of TOK I channel currents. That TOK *I-V* relationships in excised patches (Fig. 3*c*) and whole cells (Fig. 2) shift with E_K is also consistent with this hypothesis. However, that divalent cations block inward TOKI currents in excised membrane patches does not indicate that they are the only, or even the primary, mediators of rectification *in vivo*.

These data do not support the idea that ion channels endogenous to oocytes mediate TOKI currents. TOKI channels are not observed in uninjected oocytes, nor in oocytes injected with other channel cRNAs, even in the absence of external divalent cations ($n = 9$; Fig. 4*c*). The channel characterized here is observed only in patches from oocytes injected with *TOK1* cRNA. Two endogenous channels[16, 17] can be seen in patches from both control and *TOK1* cRNA-injected cells but are clearly different from TOK1 channels. The first has a small conductance (20-40 pS), similar to TOKI channels, but is insensitive to substitution of KCI by NaCI ($n = 2$), and is observed only at membrane potentials of 120 mV and above. The second has a very large conductance (> 350 pS), is activated by large potentials (±120m V), and thereafter is observed at all voltages.

A notable kinetic difference is seen between TOK1 whole-cell and patch currents. Whole-cell currents have an initial rapid phase followed by a slower second component that is apparent with high external KCl and at higher potentials (Fig. 2*a*). Patch currents show no time-dependent phase (Fig. 3*a, b*). A similar phenomenon in *eag* K^+ channels was explained by Ca^{2+}-activated chloride channel activity [18], but the slow component seen with TOKl is not altered by substitution of chloride with aspartate (Fig. 2*d*) or by removal of all Ca^{2+} from bath solutions ($n = 16$). Slow-phase current magnitude in TOKl increases with external K^+ concentration (Fig. 2*a*) and appears to reflect a process specific to K^+ ions.

There are two unusual features of TOKI channels. First, TOK I appears to be the first member of a new K + channel family defined structurally. In contrast to previously identified K^+ channel a-subunits, which bear only one pore-forming *P* region, TOKl has two. A search of the NCBI database with the TOKl coding region using the BLAST algorithm identified two hypothetical proteins in the *C. elegans* genome on chromosome III which also have a predicted structure with two *P* domains (Fig. l*c*). Second, TOKI appears to be the first example of a new functional type of K^+-selective ion channel, an 'outward rectifier' which, by analogy to the inward rectifier superfamily, passes outward K^+ current with an activation potential that is coupled to E_K . Our findings suggest that the mechanism of rectification may be similar in the two families, that is, voltage-dependent blockade. It is premature to conclude that all channels with this type of outward rectifier behaviour will contain two *P* domains in tandem, or that a structure with two *P* domains will herald only an outward rectifier K^+ channel phenotype.

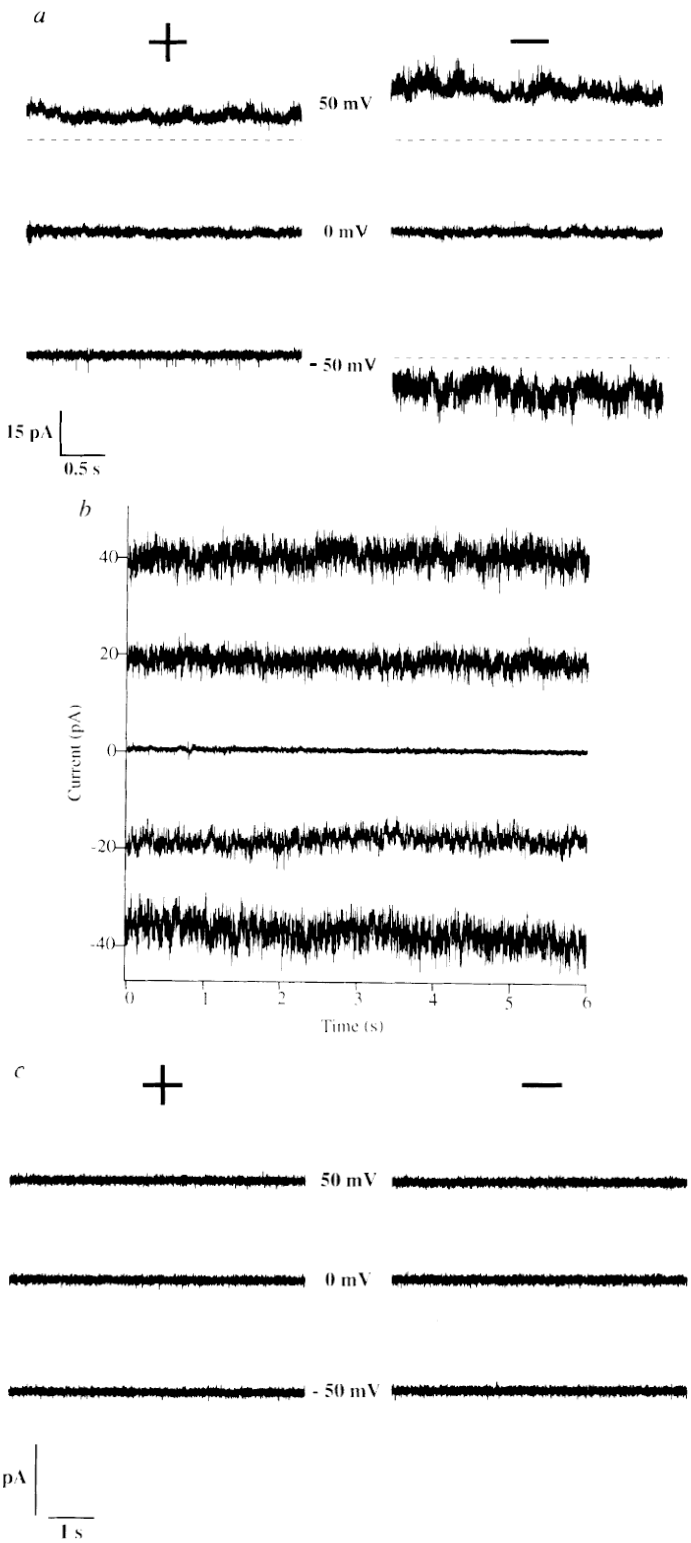


FIG. 4 Divalent-free solution at the external channel face a flows inward TOK1 currents in excised membrane patches. *a,* TOK1 channels recorded in the same excised outside-out patch with 140 mM KCI solution and divalent cations in the bath (+; in mM: 140 KCI, 1 Mg^{2+}, 0.3 Ca^{2+}, 10 HEPES, pH 7.1) and after the bath solution was exchanged for 140 mM KCI nominally divalent-free solution (–; in mM: 140 KCl, 2 K EGTA, 2 K EDTA, 10 HEPES, pH 7.1); the pipette solution was 140 mM KCl solution (in mM: 140 KCl, 1 $MgCl_2$, 5 K EGTA, 10 HEPES, pH 7.1); holding voltages were +50, 0 and –50 mV. Repetitive exchange of solutions bathing these patches showed effects to be fully reversible. *b,* TOK1 channels recorded in an excised inside-out patch with 140 mM KCI nominally divalent-free solution in the pipette and 140 mM KCI solution in the bath at +90, 50, 0, –50 and –90 mV. c, Excised outside-out patches from oocytes injected with 2 ng minK cRNA show no current under divalent-free conditions identical to a. Data were sampled as in Fig. 3.

Four α-subunits containing *P* regions associate to form one pore in *Shaker*-type channels [19, 20]. This arrangement resembles the structural organization of voltage-dependent Na^+ and Ca^{2+} channel α-subunits in which four homologous domains are repeated, each contributing one *P*-like region. The presence of two P domains in TOKl suggests that it may function as a dimer. Equally provocative is the hydropathy analysis of TOK1, which is crudely reminiscent of a *Shaker*-like channel (with P_1 placed between S5 and S6) attached to an inward rectifier-like channel (with P_2 positioned between S7 and S8) (Fig. l*b*). Protein biochemistry will be required to evaluate the stoichiometry and membrane topology of TOK1 channels. In conclusion, ORF 10911 from *S. cerevisiae* chromosome X encodes a functional protein with two *P* domains that behaves as an outward rectifier K^+ channel whose activation is coupled to E_K; it has been given the name TOK1.

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